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SHORT COMMUNICATION

AN IMPROVED MICROMETHOD FOR INFECTIVITY ASSAYS AND NEUTRALIZATION TESTS OF DENGUE VIRUSES

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An improved micromethod for infectivity assays and neutralization (N) tests of dengue (DEN) type 1–4 viruses was developed, using 96-well plates and the PAP (peroxidase-antiperoxidase) staining technique. The foci formed on BHK-21 cell monolayers in wells of the plate were readily countable under an ordinary stereomicroscope. This micromethod has the advantages over the micromethod of the Lab-Tek 8 chamber slide system of lower cost, requirement for smaller volumes of test sera and applicability to larger number of serum specimens for N tests of DEN viruses.

In some areas in Southeast Asia, DEN and Japanese encephalitis (JE) viruses co-exist and simultaneous epidemics of JE and dengue hemorrhagic fever (DHF) occur annually. However, JE and DEN viruses cannot be differentiated by the most commonly used hemagglutination inhibition (HI) test, because they are closely related and cross-react with each other serologically (Grossman et al., 1974; Melnick, 1974). The most specific serological test available is the N test (Hammon et al., 1964), but this test is much more complicated and time-consuming than HI test. Recently, Okuno et al. (1985) reported an improved rapid micromethod for the N test of JE virus using 96-well plates instead of Lab-Tek 8 chamber slides. In this paper we report a similar rapid micromethod for infectivity assays and N tests of DEN viruses.

DEN type 1 (Hawaian strain), type 2 (New Guinea B strain), type 3 (H-87 strain) and type 4 (H-241 strain) viruses were propagated in suckling mouse brains and from these brains 10% homogenates were prepared in Eagle's minimum essential medium (MEM) supplemented with 2% fetal calf serum (FCS) and stored at −80 C until used. BHK-21 cells were grown with MEM containing 10% calf serum. About 4×10^4 cells were dispensed into each well of 96-well flat bottom

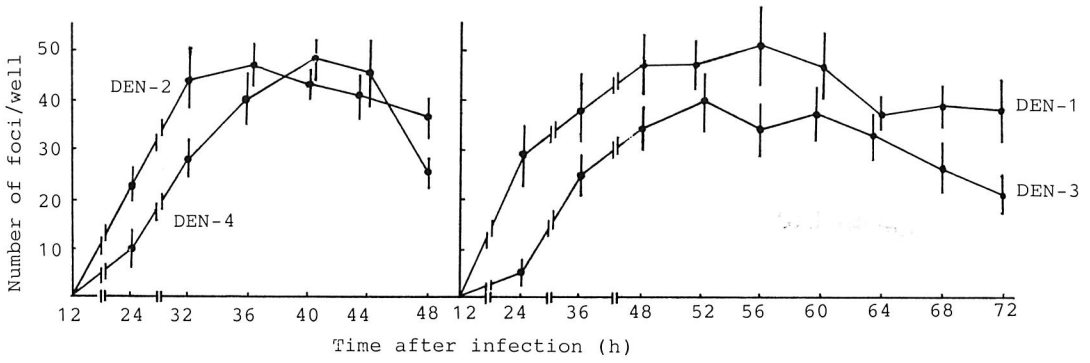

FIGURE 1. Numbers of foci of DEN type 1-4 viruses formed in wells of 96-well plates at various times after infection. The infected BHK-21 cells were fixed with methanol from 12 to 72 h after infection and stained by the PAP technique.

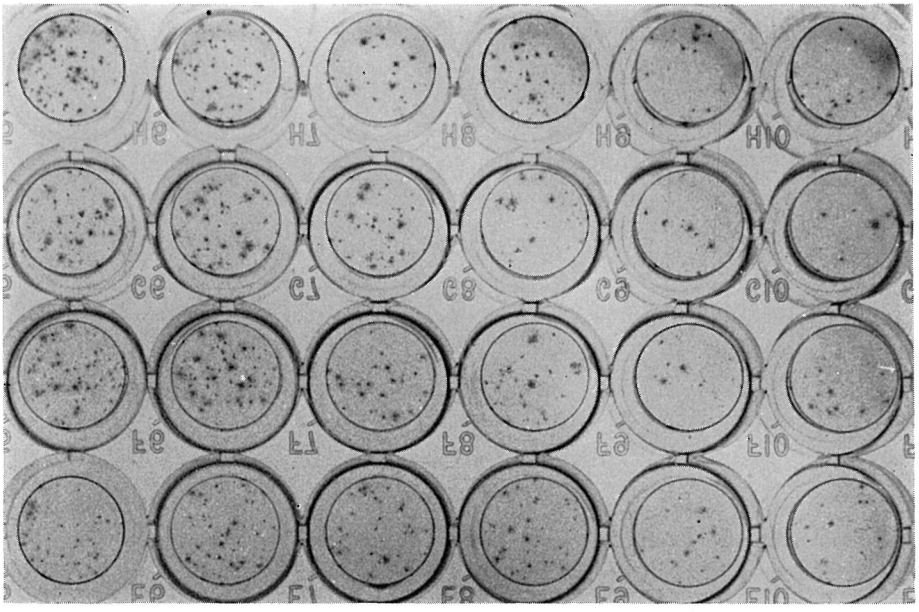

FIGURE 2. Foci formed by DEN type 2 virus at dilutions of $1:10^4$ (left two rows), $1:2\times10^4$ (middle two rows) and $1:4\times10^4$ (right two rows) stained by the PAP technique in 96-well plate. $\times 2.2$

plates (Falcon 3072, Becton Dickinison Lab., Calif., U.S.A.) and incubated in a CO_2 incubator at 37 C for 24 h. Volumes of 25 μl of serial dilutions of stock DEN viruses were inoculated onto the BHK-21 monolayers formed in each well. After adsorption for 2 h, the cells were covered with maintenance medium [MEM supplemented with 1% tragacanth gum (Nakarai Chemicals, Kyoto, Japan) and 2% FCS]. At various times after infection, the cells were rinsed with phosphate buffered saline (PBS, pH 7.4) and fixed with absolute methanol at room temperature for 20 min. The foci formed in the wells were stained by the PAP method described by Okuno et al. (1977). Briefly in-

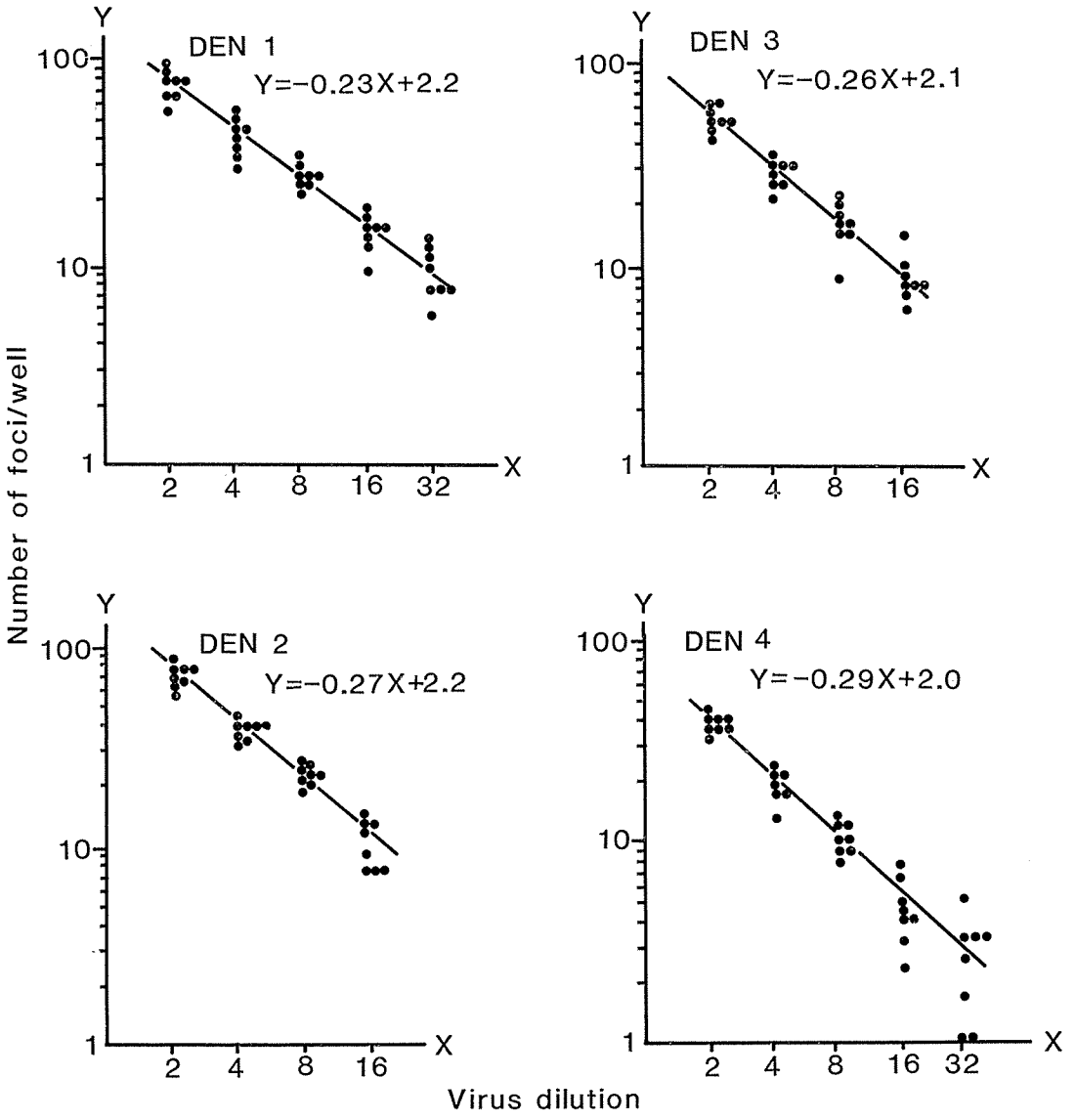

FIGURE 3. Relationship between the focus numbers and virus dilutions in the 96-well plate system ($Y = \log_{10} y$, $X = \log_2 x$).

fected cells were treated first with rabbit anti-DEN serum (1:1,000), then with sheep anti-rabbit IgG (1:1,000, Cappel Lab., Pa., U.S.A.) and finally with PAP rabbit IgG complex (1:1,000, Cappel Lab.,). Each immunological reaction was performed for 40 min at room temperature and the cells were rinsed twice with PBS after each reaction. DEN virus antigens in the infected cells were finally located by treatment with 3, 3′-diaminobenzidine tetrahydrochloride (BDH Chemicals, Pools, England) and 0.01% H_2O_2

TABLE 1. *Comparison of N titers obtained by 96-well plate and Lab-Tek 8-chamber slide systems*

N titers to DEN-1 virus

Serum specimen	8-chamber slide	96-well plate
R- 20	2.06[a]	2.18[a]
27	2.30	2.02
37	2.68	2.40
57	<1.00	<1.00
61	1.51	1.53
78	2.26	2.04
83	2.79	2.90
87	2.28	2.30
89	2.11	2.18
101	<1.00	<1.00
109	1.83	1.78
111	1.65	1.48
137	2.49	2.34
138	2.59	2.53
149	<1.00	<1.00

N titers to DEN-3 virus

Serum specimen	8-chamber slide	96-well plate
S- 69	1.95[a]	1.87[a]
70	3.38	3.11
71	2.15	1.90
74	1.56	1.49
76	1.96	1.90
79	<1.00	<1.00
81	1.65	1.56
85	1.00	1.00
96	2.30	2.23
97	2.18	2.04
104	<1.00	<1.00
106	1.90	1.85
115	1.95	1.76
116	1.76	1.65
119	2.30	2.26

N titers to DEN-2 virus

Serum specimen	8-chamber slide	96-well plate
L- 72	1.38[a]	1.15[a]
82	1.36	1.48
88	1.56	1.48
108	1.79	1.85
110	1.60	1.41
119	2.70	2.70
123	2.00	1.85
125	2.54	2.54
128	2.38	2.53
129	1.90	1.95
131	1.93	1.94
132	1.41	1.51
134	1.65	1.58
142	1.65	1.60
155	1.78	1.90

N titers to DEN-4 virus

Serum specimen	8-chamber slide	96-well plate
L- 50	1.81[a]	1.70[a]
73	2.08	2.08
90	2.11	2.26
93	1.00	1.20
96	1.32	1.30
135	2.00	1.88
154	1.20	1.30
156	1.70	1.60
158	2.00	2.08
159	2.19	2.30
161	2.48	2.49
164	2.11	2.18
R-156	1.70	1.11
166	<1.00	<1.00
167	1.70	1.70

[a] Log_{10}

in PBS for 10 min. Then, the cells were washed with tap water and dried. The foci formed in the wells were examined under a stereomicroscope.

As shown in Fig. 1, the foci of all four DEN viruses were recognized first as clusters of brownish cells 24 h after infection. Thereafter, in cell monolayers infected with DEN type 2 and 4 viruses the numbers of foci increased until 36 h and then remained con-

stant until 44 h, while in those infected with DEN type 1 and 3 viruses, they increased slightly until 48 h and then remained constant until 60 h. After these time, the number of foci decreased due to overlapping of the foci. Therefore, the incubation periods after infection used for focus staining were 40 h for DEN type 2 and 4 viruses and 56 h for DEN type 1 and 3 viruses. Fig. 2 shows the foci of DEN type 2 virus stained by the PAP technique in a 96-well plate. The eight wells in the left two rows were inoculated with DEN type 2 virus at a dilution of $1:10^4$ and the number of foci in each of these eight wells were countable. It was difficult to count foci when their number was more than 80 per well. The average diameter of foci was 0.1 mm. The foci of DEN type 1, 3 and 4 viruses were similar to those of DEN type 2 virus in size and shape when stained at the optimal time after infection. The number of foci in a well was countable in one field of view under a stereomicroscope. Plots of focus number against virus dilution were linear for all the DEN viruses, as shown in Fig. 3, indicating that 96-well plate system can be used for infectivity assays. The least squares regression lines for DEN type 1, 2, 3 and 4 viruses were calculated to be $Y=-0.23X+2.2$, $Y=-0.27X+2.2$, $Y=-0.26X+2.1$ and $Y=-0.29X+2.0$, respectively.

For N tests of DEN type 1–4 viruses by the 96-well plate system, human sera obtained in Thailand were serially diluted 4 fold from 1: 20 with MEM supplemented with 2% FCS and 50 μl samples of each dilution were introduced into 96-well roud bottom plates (Terumo, Tokyo, Japan). An equal volume of DEN virus suspension adjusted to 80 focus forming units (FFU) per well was mixed with each dilution of test serum. Then, the serum-virus mixtures were incubated for 2 h at 28 C and 25 μl of each mixture was inoculated in duplicate onto BHK-21 cells in wells of a 96-well plate using a multichannel pipette (Titertek, Flow Lab., Finland). Fixation and staining by the PAP technique were followed as above. N tests of DEN viruses in the 8 chamber slide (Miles Lab., Ill., U.S.A.) system were carried out by the method of Okuno et al. (1978). The N antibody titers were expressed as reciprocal of the highest serum dilution showing 50% focus reduction. Table 1 shows that there was good correspondence between the N titers determined by the 96-well plate and 8 chamber slide systems. These results indicate that the 96-well plate system can be used instead of 8 chamber slide system for N tests of DEN viruses.

N tests of DEN viruses by the ordinary plaque reduction method require long incubation periods (7–14 days), and relatively large volumes of test sera. Some semi-micro and micro plaque reduction N tests for DEN viruses have been reported (Fujita et al., 1975; Morens et al., 1985a, b), but the incubation periods are not much shorter. In the present study we used 96-well plates and the PAP staining technique for infectivity assays and N tests. This method greatly shortens the incubation periods after infection and requires smaller volumes of cells, media and especially sera for N tests with the use of 96-well plates, so that N tests for DEN viruses can be made on a larger number of serum specimens in a single experiment. This micromethod will be useful for serodiagnosis of DHF patients and for seroepidemiological studies in areas where DEN and JE viruses co-exist.

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